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THE EFFECT OF BENZODIAZEPINES AND β-CARBOLINES ON GABA-STIMULATED CHLORIDE INFLUX BY MEMBRANE VESICLES FROM THE RAT CEREBRAL CORTEX

Takaaki Obata and Henry I. Yamamura

Department of Pharmacology, College of Medicine,
University of Arizona, Tucson, Arizona 85724

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Benzodiazepine agonists such as diazepam, flunitrazepam and clonazepam enhanced GABA (30 μM)-stimulated $^{36}Cl^-$ uptake in membrane vesicles from the rat cerebral cortex. The rank order of potencies was flunitrazepam > diazepam = clonazepam. β-Carboline-3-carboxylate esters β-CCM, β-CCE and DMCM inhibited GABA-stimulated $^{36}Cl^-$ uptake. The rank order of inhibitory potencies was DMCM > β-CCM > β-CCE. The benzodiazepine antagonist Ro15-1788 antagonized the enhancement of flunitrazepam and the inhibition of DMCM on GABA-stimulated $^{36}Cl^-$ uptake in a competitive inhibitory manner. These results suggest that benzodiazepine receptors regulate GABA-stimulated $^{36}Cl^-$ uptake and there is a functional coupling between the GABA and benzodiazepine receptors, and chloride channels in membrane vesicles from the rat cerebral cortex.

The benzodiazepines are widely used as anxiolytics, sedative-hypnotics, muscle relaxants and anticonvulsants. Their pharmacological effects appear to be mediated by enhancing the actions of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (1, 2). Further, the discovery of specific high affinity binding sites for benzodiazepines on brain membranes (3, 4) led to the existence of the GABA/benzodiazepine receptor complex, which is coupled to the GABA-gated chloride channel. The chloride channel is also allosterically regulated by barbiturates, picrotoxin and cage convulsants (5, 6). While receptor binding studies have indicated an allosteric interaction between the benzodiazepine and GABA receptors (6, 7) and an allosteric interaction of [^{35}S]t-butylbicyclophosphorothionate (TBPS) binding to the chloride channel by benzodiazepines and β-carbolines (8, 9, 10), it has not yet been shown biochemically whether benzodiazepines functionally regulate GABA-gated chloride channels. Recently, a useful biochemical technique to measure GABA-stimulated $^{36}Cl^-$ uptake in mouse brain membrane vesicles was reported and it

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was shown that GABA and barbiturate receptors were functionally coupled with chloride channels (11). Using this system, we now report that benzodiazepines and β-carbolines regulate GABA-stimulated $^{36}Cl^-$ uptake in membrane vesicles from the rat cerebral cortex.

METHODS

Preparation of membrane vesicles Membrane vesicles from the rat cerebral cortex were prepared by the procedure of Harris and Allan (11) with minor modifications. Male Sprague-Dawley rats (200-250 g) were decapitated and their brains were removed. Cerebral cortices were rapidly dissected and homogenized by hand (12 strokes) using a glass-glass homogenizer in 10 vol (w/v) of ice-cold buffer (145 mM $NaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$ and 10 mM HEPES adjusted to pH 7.5 with Tris base). The homogenate was centrifuged at 1000 x g for 15 min at 4°C. The supernatant was decanted and the pellet resuspended in 10 vol (w/v) of buffer was centrifuged at 1000 x g for 15 min at 4°C. The final pellet was resuspended in buffer to a final protein content of 8-9 mg/ml. Protein concentrations were determined by the method of Lowry et al. (12).

Measurement of $^{36}Cl^-$ uptake Aliquots of membrane vesicle suspension (200 µl) were preincubated for 10 min at 30°C. After preincubation, $^{36}Cl^-$ uptake was initiated by the addition of 200 µl solution containing $^{36}Cl^-$ (0.2 µCi/ml) and GABA (30 µM). After incubation for 3 sec, uptake was terminated by the addition of ice-cold buffer (4 ml x 2) followed by rapid vacuum filtration (0.05 % polyethylenimine treated Whatman GF/C glass microfiber filter) with a Hoefer Scientific filter manifold. Filters were washed with ice-cold buffer (1 ml x 10) and $^{36}Cl^-$ content of the filters was determined by liquid scintillation counting. Benzodiazepines and β-carbolines were preincubated with membrane vesicles at 30°C for 10 min. GABA-stimulated $^{36}Cl^-$ uptake was calculated as difference between basal uptake in the absence of GABA and total uptake in the presence of GABA. All data represent the mean ± SEM of percentage of $^{36}Cl^-$ uptake stimulated by 30 µM of GABA.

RESULTS

The uptake of $^{36}Cl^-$ into membrane vesicles from the rat cerebral cortex was stimulated by 30 µM of GABA (basal uptake : 11.01 ± 0.54, GABA-stimulated uptake : 22.45 ± 1.00, net stimulated uptake : 11.44 ± 0.58 nmoles/mg protein/3 sec ; n = 20).

Diazepam, flunitrazepam and clonazepam enhanced GABA (30 µM)-stimulated $^{36}Cl^-$ uptake in a concentration-dependent manner (Fig. 1). The maximal effect of these benzodiazepines was observed at 10^{-6} to 10^{-5} M. Diazepam and clonazepam showed similar enhancement at 10^{-5} M with a 30.0 and 31.0 % enhancement of GABA-stimulated $^{36}Cl^-$ uptake, respectively. Flunitrazepam produced a 47.7 % enhancement of GABA-stimulated $^{36}Cl^-$ uptake at 10^{-5} M. The EC_{50} value for flunitrazepam was 2.3 x 10^{-8} M.

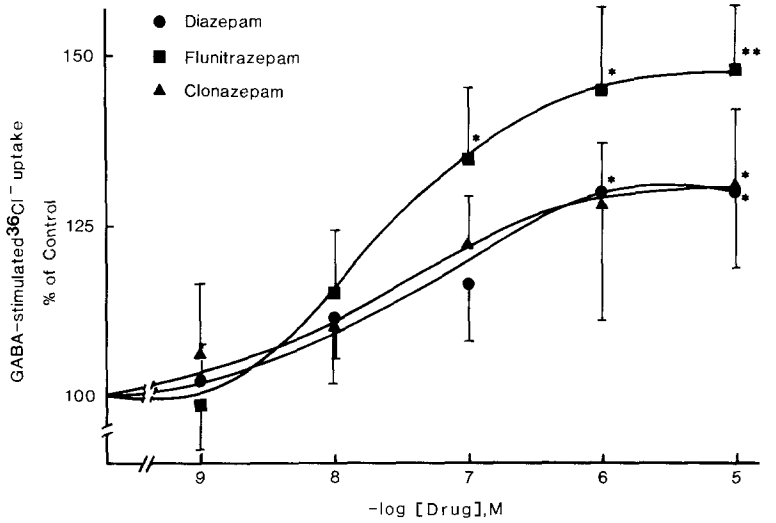

Fig. 1. The effect of diazepam, flunitrazepam and clonazepam on GABA- (30 μM)-stimulated $^{36}Cl^-$ uptake in membrane vesicles from the rat cerebral cortex. Each point represents the mean ± SEM of five different preparations.
*, **) Significantly different from control at $p<0.05$ and $p<0.01$, respectively.

Methy-β-carboline-3-carboxylate (β-CCM), ethyl-β-carboline-3-carboxylate (β-CCE) and 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylic acid methyl ester (DMCM) inhibited GABA-stimulated $^{36}Cl^-$ uptake in a concentration-dependent manner (Fig. 2). The maximal inhibition of these β-carbolines was observed at

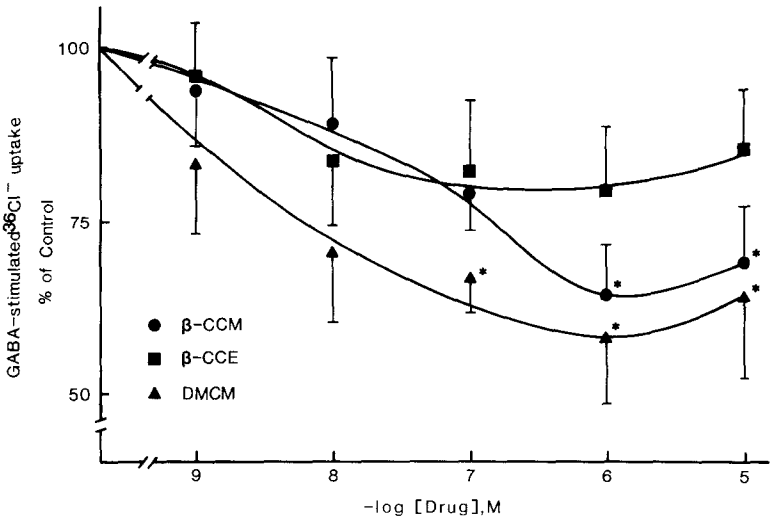

Fig. 2. The effect of β-CCM, β-CCE and DMCM on GABA (30 μM)-stimulated $^{36}Cl^-$ uptake in membrane vesicles from the rat cerebral cortex. Each point represents the mean ± SEM of five different preparations.
*) Significantly different from control at $p<0.05$.

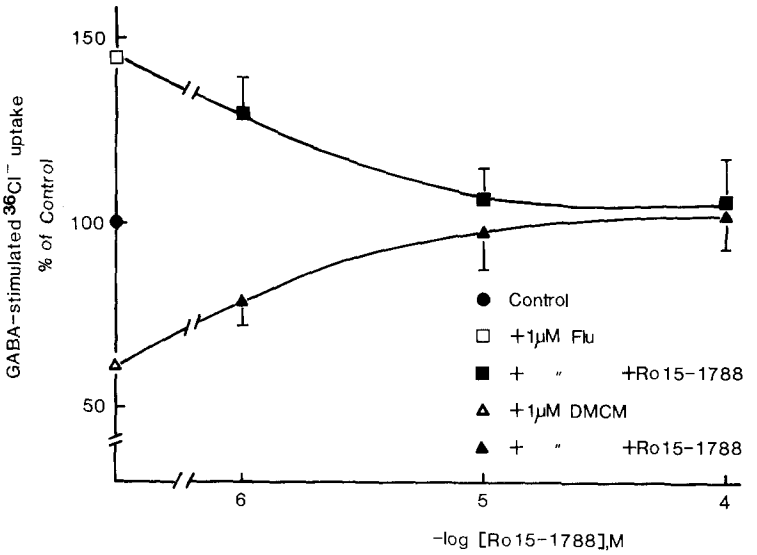

Fig. 3. The antagonism by Ro15-1788 of GABA (30 μM)-stimulated $^{36}Cl^-$ uptake enhanced by flunitrazepam and inhibited by DMCM. Each point represents the mean ± SEM of five different preparations.

10^{-6} M. DMCM was the most potent inhibitor with 42.1 % inhibition on GABA-stimulated $^{36}Cl^-$ uptake. The IC_{50} value for DMCM was 3.0×10^{-9} M.

Ro15-1788, a benzodiazepine antagonist, blocked the enhancement of 1 μM flunitrazepam and the inhibition of 1 μM DMCM on GABA-stimulated $^{36}Cl^-$ uptake in a concentration-dependent manner (Fig. 3).

DISCUSSION

Using a membrane vesicle preparation from the rat cerebral cortex, we examined the effects of several benzodiazepines and β-carbolines on the GABA-stimulated $^{36}Cl^-$ uptake. Diazepam, flunitrazepam and clonazepam which are benzodiazepine agonists enhanced the activity of GABA on $^{36}Cl^-$ uptake. On the other hand, β-CCM, β-CCE and DMCM which are inverse benzodiazepine agonists inhibited the GABA-stimulated $^{36}Cl^-$ uptake.

In preliminary experiments we confirmed that GABA (3-300 μM) stimulated $^{36}Cl^-$ uptake in membrane vesicles from the rat cerebral cortex in a concentration-dependent manner (data not shown). In addition, 100 μM of pentobarbital enhanced GABA (30 μM)-stimulated $^{36}Cl^-$ uptake by 77.2 % of control(data not shown). These findings indicate that the membrane vesicle

preparation from the rat cerebral cortex contains GABA and barbiturate receptors which are functionally coupled to the chloride channels as reported in mouse brain (11).

We now report that benzodiazepine agonists which exhibit anxiolytic and anticonvulsant activities show an enhancement on GABA-gated chloride channels and that β-carbolines which have proconvulsant or convulsant activity show an opposite effect on this biochemical response. These findings correlate with the pharmacological actions of the benzodiazepines and β-carbolines in the mammalian brain.

Flunitrazepam was the most potent benzodiazepine stimulator and DMCM the most potent β-carboline inhibitor of the drugs examined on the GABA-gated chloride channels. These results correlate with the GABA-shift ratios which have been obtained from radioligand binding studies which show that GABA enhances the affinity of benzodiazepine agonists and decreases the affinity of inverse benzodiazepine agonists for the benzodiazepine receptors (13).

The effects of flunitrazepam and DMCM on the GABA-stimulated $^{36}Cl^-$ uptake were blocked by Ro15-1788 in a competitive inhibitory manner. Since Ro15-1788 is a benzodiazepine antagonist, the functional effects of the benzodiazepines and β-carbolines on the GABA-stimulated $^{36}Cl^-$ uptake are mediated by the benzodiazepine receptors.

In conclusion, pharmacologically relevant benzodiazepines enhanced GABA-stimulated $^{36}Cl^-$ uptake in membrane vesicles from the rat cerebral cortex and proconvulsant or convulsant β-carbolines inhibited this functional response. The effects were antagonized by the benzodiazepine antagonist, Ro15-1788. These findings support the hypothesis that there exists a functional coupling between the GABA receptor and benzodiazepine receptor and the chloride channel, and that the benzodiazepine receptor may allosterically modulate the GABA receptor-chloride channel system.

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